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Genetic Control of Enzyme Induction in the β-Ketoadipate Pathway of *Pseudomonas putida*: Deletion Mapping of *cat* Mutations

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A number of spontaneous mutant strains of *Pseudomonas putida*, obtained by repeated selection for inability to grow with *cis,cis*-muconate, have been shown to carry deletions in *catB*, the structural gene for muconate lactonizing enzyme. These strains have been employed for deletion mapping of the genetic region containing *catB* and *catC* (the structural gene for muconolactone isomerase, the synthesis of which is coordinate with that of muconate lactonizing enzyme). All deletions that overlap mutant sites located on the left side of the genetic map, as well as the point mutations in that region, lead to a pleiotropic loss of both *catB* and *catC* activities. We propose that this region to the left of *catB* has a regulatory function. Although the details of regulation at the molecular level are unclear, our data indicate that *catB* and *catC* may well be controlled by a mechanism unlike any yet described by workers on enteric bacteria.

The β-ketoadipate pathway is a complex set of convergent reaction sequences operative in the degradation of a variety of aromatic and hydroaromatic compounds by many aerobic bacteria. Figure 1 shows those elements of the pathway possessed by *Pseudomonas putida*. The regulation of the synthesis of enzymes of this pathway has been studied in *P. putida* (11) and *P. aeruginosa* (9). The regulation of the synthesis of enzymes of the central sequences (those reaction sequences involved in the dissimilation of catechol and protocatechuic acid) was found to be identical in the two species of *Pseudomonas* (Fig. 2). The characteristic feature of the *Pseudomonas* regulatory pattern is the coordinate product induction by β-ketoadipate [or its coenzyme A thioester] of carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, enollactone hydrolase, and transferase. A consequence of this mode of regulation is the gratuitous synthesis of carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase during growth with precursors of catechol. Such gratuitous enzyme synthesis has been demonstrated in other species of *Pseudomonas* (11; R. Y. Stanier, *personal communication*); thus this pattern of regulation appears to be characteristic of the genus. Other groups of bacteria do not possess this feature of the control pattern (11), and detailed study of the regulation of this pathway in *Acinetobacter* (*Moraxella*) *calcoaceticus* (3) and in *Alcaligenes eutrophus* (8) has shown that the regulatory mechanisms are completely different. Canovas et al. (2) have proposed that the possession of different control patterns for such a complex pathway by two different groups of bacteria indicates either an evolutionary divergence of considerable antiquity or independent acquisition of the pathway.

The physiological studies of this pathway have now been augmented by genetic studies in *P. aeruginosa* (9) and *P. putida* (19). The arrangement on the chromosome of genes specifing enzymes of the central sequences is closely similar in the two species, which supports the proposal (2, 15) that the pathway and regulatory apparatus have a common evolutionary origin in the genus *Pseudomonas*. These studies, however, were addressed only to determining the linkage relationships of the genes of the β-ketoadipate pathway; no attempt was made to investigate in more detail the mechanisms of genetic regulation in *Pseudomonas*. It has been proposed (11) that those enzymes whose synthesis is coordinate are the products of expression of complex operons; the

genetic studies so far performed are consistent with that notion. One such group of enzymes is composed of muconate lactonizing enzyme (MLE) and muconolactone isomerase (MI), the last two enzymes of the catechol branch of the pathway. They are substrate-induced by *cis,cis*-muconate (Fig. 2). Preliminary mapping has shown that the genes governing their synthesis (*catB* and *catC*) lie in close proximity to one another on the chromosome (19).

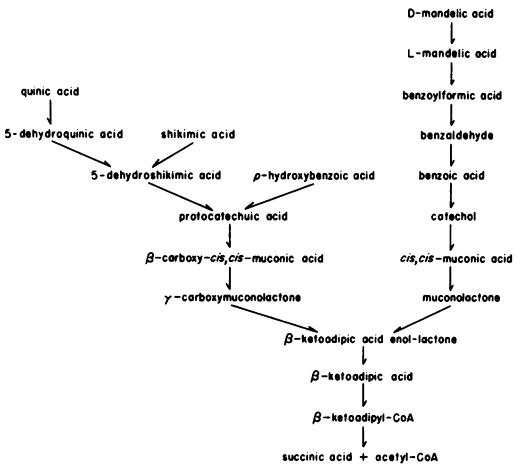

FIG. 1. *Convergent reaction sequences of the β-ketoadipate pathway in Pseudomonas putida.*

We present here a more detailed genetic study of this region of the chromosome and describe the identification and mapping of numerous point mutations and deletions in the *catB* region. In addition, we present evidence indicating that mutations confined to one region of the map apparently affect the regulation of both *catB* and *catC* expression.

MATERIALS AND METHODS

Biological materials. *Pseudomonas putida* mutant strains utilized in this study are shown in Table 1. All were derived from PRS1 (strain 90 of reference 16). The isolation of spontaneous mutants with penicillin G and D-cycloserine counterselection has been described by Ornston et al. (12). Mutants induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were obtained as described by Wheelis and Stanier (19). The transducing phage employed was pf16·h2 (4).

Transductions. Since recombination between a point mutation and a deletion within a single cistron is a comparatively rare event, it was necessary to modify the standard procedures (4) for *Pseudomonas putida* transductions. Thus, 5×10^9 phage were mixed with 2.5×10^{10} recipient bacteria in a final volume of 3.5 ml. After 30 min of incubation at 30 C with gentle shaking, the mixture was centrifuged and the pellet was suspended in 0.2 ml of anti-pf16 serum ($K = 10\ min^{-1}$). After an additional 15 to 30 min of incubation at room temperature, 0.1-

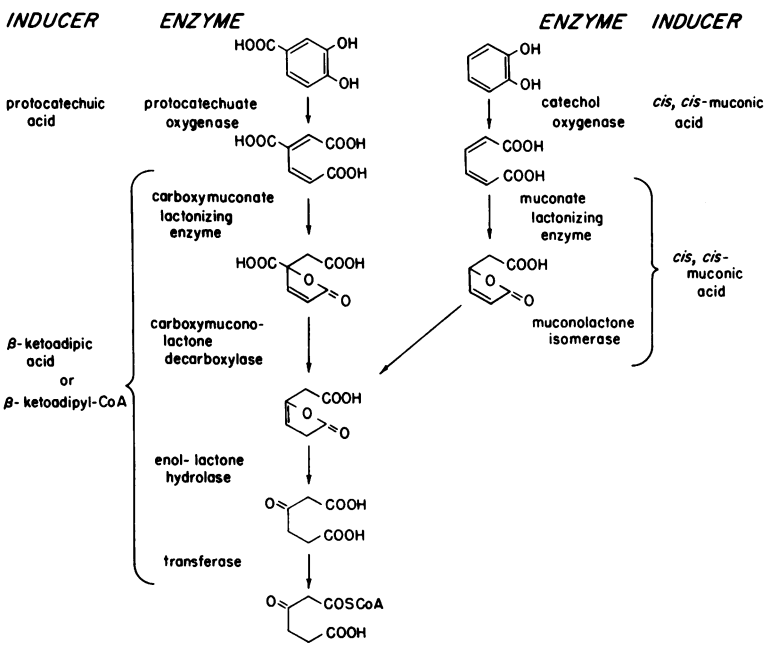

FIG. 2. *Regulation of synthesis of enzymes of the central sequences of the β-ketoadipate pathway in Pseudomonas putida. Brackets denote coordinate synthesis.*

TABLE 1. *Bacterial strains used*

Strain	Genotype[a]	Parent	Derived by[b]	Revertant frequency	Activity		Reference
					MLE	MI	
PRS1	Wild				+	+	(19)
PRS2	*mdlA1001*	PRS1	UV		+	+	(19)
PRS4	*catB1001*	PRS1	NMG	4×10^{-10}	−	+	(19)
PRS6	*pcaB1002*	PRS1	NMG		+	+	(19)
PRS7	*mdlA1001, catB1002*	PRS2	EMS	3×10^{-8}	−	+	
PRS9	*mdlA1001, catC1004*	PRS2	EMS	5×10^{-10}	+	−	(19)
PRS40	*pcaB1002, catC1006*	PRS6	NMG	1.3×10^{-9}	+	−	(19)
PRS43	*catB1009*	PRS1	NMG	4×10^{-9}	−	+	
PRS61	*catB1010*	PRS1	NMG	5×10^{-10}	−	+	
PRS66	*catB1011*	PRS1	NMG	5×10^{-9}	−	+	
PRS67	*catB1012*	PRS1	NMG	2×10^{-9}	−	+	
PRS71	*cat-1013*	PRS1	NMG	9.5×10^{-10}	−	−	
PRS73	*cat-1015*	PRS1	NMG	7×10^{-8}	−	−	
PRS74	*catB1014*	PRS1	NMG	3×10^{-9}	−	+	
PRS524	*cat-1016*	PRS1	NMG	9.5×10^{-10}	±	−	
PRS2000	*per-1103*	PRS1	S		+	+	
PRS2001	*catB1109, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	+	
PRS2002	*cat-1110, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	−	
PRS2003	*cat-1111, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	−	
PRS2004	*cat-1112, per-1103*	PRS2000	S-PCS	3.5×10^{-10}	−	−	
PRS2005	*catB1113, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	+	
PRS2006	*catB1114, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	+	
PRS2007	*catB1115, per-1103*	PRS2000	S-PCS	1.2×10^{-9}	−	+	
PRS2008	*catB1116, per-1103*	PRS2000	S-PCS	1×10^{-9}	−	+	
PRS2009	*catB1117, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	+	
PRS2010	*catB1118, per-1103*	PRS2000	S-PCS	2.2×10^{-9}	−	+	
PRS2011	*catB1119, per-1103*	PRS2000	S-PCS	5×10^{-10}	−	+	
PRS2012	*cat-1120, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	−	
PRS2013	*catB1121, per-1103*	PRS2000	S-PCS	5×10^{-10}	−	+	
PRS2014	*cat-1122, per-1103*	PRS2000	S-PCS	2.5×10^{-11}	−	−	
PRS2015	*catB1123, per-1103*	PRS2000	S-PCS	$<5 \times 10^{-11}$	−	+	

[a] Genotype symbols: *mdl* and *pca*, mutations in reaction sequences of the β-ketoadipate pathway not relevant to this study; *per*, permeable to *cis,cis*-muconate; *catB*, lacks muconate lactonizing enzyme (MLE); *catC*, lacks muconolactone isomerase (MI); *cat-*, lacks both MLE and MI.

[b] Abbreviations: UV, induced by ultraviolet irradiation; NMG, induced by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; EMS, induced by treatment with ethylmethanesulfonate; *S*, spontaneous; PCS, counterselected with penicillin G and D-cycloserine.

ml samples were spread on benzoate plates. The plates were incubated at 30 C for 3 to 4 days and then were examined for colonies (19). All crosses were performed at least four times. When phage propagated on the wild type (PRS1) was used, about 1,000 recombinants were obtained per 10^{10} input phage.

Growth of bacteria and preparation of lysates. Bacteria were grown on medium E of Vogel and Bonner (18) for use as recipients in transductions and on the medium of Stanier et al. (16) for all other purposes.

Lysates were prepared in the preservative medium (PM) of Niblack (Ph.D. thesis, Univ. of Illinois, Urbana, 1968) by the agar overlay technique as described by Adams (1).

Preparation of cell-free extracts and enzyme assays. Cell-free extracts were prepared by sonic treatment as described by Ornston and Stanier (13). Published spectrophotometric assays were used for measurement of the following enzymes: catechol-1,2-oxygenase (pyrocatechase) (5); MLE and MI (10).

Serology. The production of cross-reacting material (CRM) was determined by double-diffusion experiments with antisera prepared as described by Stanier et al. (17), except that, in the case of pleiotropic negative mutants (those lacking both MLE and MI activity), it was not possible to determine the amount of crude extract to add to an antigen well by enzyme assay. In such cases, an amount of crude extract was added to the well such that the amount of protein added was the same as for the wild type.

Conditions of growth and induction. Inducing conditions were achieved by growing cells for three generations with 10 mM succinate and 5 mM benzoate. The intracellular production of the inducer, *cis,cis*-muconate, was established by the assay of catechol oxygenase which is induced by this compound (11).

RESULTS

Reversion rates. Fifteen spontaneous mutants were isolated by repeated selection with penicillin G and D-cycloserine for strains unable to grow at the expense of *cis,cis*-muconate (12). The strains were deficient either in MLE activity or in both MLE and MI activity (Table 1). These and similar mutants previously isolated with no counterselection after chemical mutagenesis were tested for their ability to revert spontaneously. Table 1 includes the results of these tests. All 12 induced mutants and 7 of the 15 spontaneous mutants accumulated a detectable population of revertants and thus presumably contain single-site mutations. Spontaneous revertants of the eight remaining mutants have not been observed, although over 10^{11} bacteria have been plated in the course of this investigation; we conclude that these mutants carry deletions. All mutants are able to convert benzoate to *cis,cis*-muconate and thus are able to synthesize both benzoate oxidase and catechol oxygenase.

Serology. All the *cat* mutants described in Table 1 were tested for the production of CRM by the Ouchterlony double-diffusion technique. Only two mutations, *catB1117* and *catB1121*, led to the production of CRM for an inactive enzyme. In all other mutants, the loss of an enzyme's activity was accompanied by the loss of CRM.

Genetic mapping. The inability of eight of the spontaneous mutants to revert led us to believe that they contain large deletions. These mutants therefore were used as recipients in crosses with each of the 19 point mutants as donors (Table 2). In each case, one or more of the crosses failed to yield recombinants, and two of the recipients (PRS2002, PRS2003) appear to have most of *catB* deleted. None of the deletions, however, overlaps the sites of either of the two *catC* mutations. These results support the conclusion that the nonreverting mutants harbor deletions.

DISCUSSION

We have isolated 27 mutants lacking MLE, MI, or both. Of these, eight failed to revert spontaneously and thus were tentatively classified as deletion mutants. All eight failed to yield recombinants with one or more point mutants, which supports the classification of their lesions as deletions. On the basis of these crosses, a unique and internally consistent genetic map of this region can be constructed (Fig. 3). The high frequency of deletion mutants among the spontaneous mutant strains (8 of 15) indicates that the enrichment of such mutants with penicillin G and D-cycloserine (12) may provide a useful general technique for the isolation of deletion mutants in *Pseudomonas*.

Of the 27 single-site and deletion mutants studied, only two produce CRM for an enzyme that they lack. The lesion in one (*catB1121*) is at the extreme "left" end of *catB;* that of the other (*catB1117*) is a short deletion near the right end of the same gene. Since the gene product of *catB* appears to have a molecular weight of about 40,000 (R. B. Meagher and L. N. Ornston, *unpublished data*), the paucity of CRM-producing mutants is surprising. A possible explanation is that the subunits themselves, of which there are probably five to six in the native enzyme (molecular weight about 220,000; reference 10), are neither antigenically nor enzymatically active, and that aggregation of the subunits is extremely sensitive to any conformational alteration such as might be expected in the mutant strains. This would be consistent with the fact that both CRM-pro-

TABLE 2. *Number of wild-type recombinants per 10^{10} phage*[a]

Recipient	Donor																		
	—1013	*—1015*	*—1016*	*—1112*	*—1122*	*B1001*	*B1002*	*B1009*	*B1010*	*B1011*	*B1012*	*B1014*	*B1115*	*B1116*	*B1118*	*B1119*	*B1121*	*C1004*	*C1006*
B1109	30	4	44	18	34	4	2			6	3		2	4			1	30	4
—1110							0.5				2							2	11
—1111																		12	2
B1113	10		10	14	12		2				2							6	9
B1114	20	4	28	18	14	1	7		2	2	1		2	6		2	1	26	2
B1117	6	3	2	14	6	2	1	4	2	2	2		4	7	3	2	2	52	4
—1120	4		4		2	26	2	1	6	2	8	4	42	20	2	2		10	11
B1123	43	14	14	23	18	4	12							3			2	20	7

[a] Blanks indicate fewer than 0.5 recombinants per 10^{10} phage.

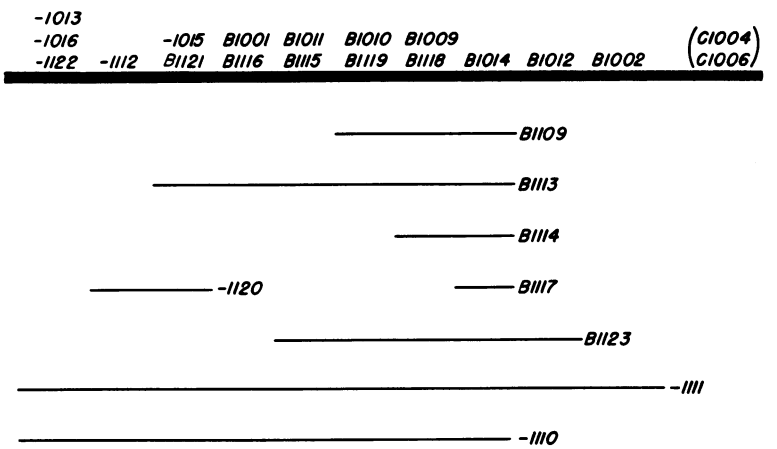

FIG. 3. *Tentative genetic map of the catB region. Markers in catC are enclosed in parentheses to indicate that their position to the right of catB is uncertain. Point mutants (above the heavy line) do not yield recombinants in crosses with deletion mutants (below the heavy line) which cover them.*

ducing mutants show identity with the wild type; no spurring was observed. Since only two *catC* mutants were examined, no conclusions can be drawn about the frequency of CRM-producing mutants in that gene.

The deletion map should be regarded as tentative, despite its internal consistency. It is possible, even probable, that the deletions are shorter than indicated, since some point mutants may well have yielded no recombinants owing to their proximity to, but not coincidence with, a deletion. For instance, evidence discussed below indicates that deletion *cat-1120* most probably does not cover *catB1121*. In theory, however, the order deduced for point mutations should not be altered by this observation since each mutant site should be equally affected. That most of the deletions are as extensive as we show them or that there is a strict proportionality between recombinant yield and distance between point mutation and deletion end point is supported by the agreement between two-point crosses and the deletion map (21). We therefore feel that the map of *catB* point mutations as presented is reasonably accurate. Since none of the deletions covered *catC*, however, its placement to the right of *catB* in Fig. 3 is arbitrary.

Our most interesting finding is the presumptive definition of a genetic region with a regulatory function to the "left" of *catB*. All point mutations in this area abolish simultaneously the expression of *catB* and *catC*. Such a pleiotropic effect could be the result either of polarity effects or of a mutation in a regulatory element. The former explanation seems unlikely in view of the phenotype of PRS2200 (21). This strain was selected as an induced revertant of PRS2012 (which by transductional analysis appears to carry a deletion in the area in which are found the pleiotropic mutants). This strain (PRS2200) has both MLE and MI proteins which appear to be identical with those of the wild type, thus making unlikely the supposition that it is a revertant of a polar deletion. In addition, MLE and MI are synthesized constitutively, a fact which supports the idea that this region contains a regulatory gene of some type. We thus propose the genetic designation *catR* for the pleiotropic negative point mutants and the deletion carried by PRS2012. Such a designation should be regarded as tentative until such time as complementation analysis confirms that all the mutations lie within a single cistron.

Assuming that the inferences drawn above are correct, it is interesting to speculate about the nature of such a regulatory gene. All primary mutations which we have mapped into this area are pleiotropic negative mutants. These include five point mutants and one deletion. A constitutive mutant has been derived only as the result of a secondary mutation in a *catR* strain. Thus it is highly unlikely that MLE and MI form an operon under classical negative control (7). Such a hypothesis would necessitate supposing that in this system the occurrence of i^s type mutations (20) is a frequent event, since 5 of 19 point mutants isolated in this region showed such a phenotype. It is also difficult to imagine how a deletion mutant could be phenotypically i^s. In the light of current knowledge of protein structure and specificity, such an idea seems intrinsically unappealing.

A more attractive model for this system is that of positive control (14). In such a system, most mutants in the regulatory gene would be

expected to be pleiotropic negatives rather than constitutives. The occurrence of constitutive revertants of PRS 2012 is also readily explicable by this model, although the inducible revertants are more difficult to account for. An additional discrepancy is the lack of temperature-sensitive pleiotropic mutants in this system. Irr and Englesberg (6) found that a high proportion of *araC*$^-$ mutants are able to grow with arabinose at low temperature and thus are temperature-sensitive. Furthermore, an *araC*$^-$ mutant was found to yield temperature-sensitive revertants if selected for reversion at low temperature. None of the *catR* mutants we have isolated at 30 C is able to grow at 22 C, and none of 269 revertants isolated from various of these mutants at 22 C proved temperature-sensitive upon shift to 31 C (M. L. Wheelis, *unpublished data*).

Alternatives to the idea that the *catR* mutations lie in a regulatory gene (e.g., they are at the site of ribonucleic acid polymerase binding to deoxyribonucleic acid or of ribosome binding to messenger ribonucleic acid) are not attractive for the same reason that the classical operon model is not; the frequency with which mutants bearing this class of mutations occurs is far higher than would be predicted on the basis of our present understanding of the mechanism of protein synthesis.

Our results thus indicate that it is too early to make assertions, with any certainty, about the nature of control of gene expression in *Pseudomonas*. The mechanisms by which enzyme synthesis is regulated in this group of prokaryotes may well turn out to be novel in terms of the models developed by those studying the enteric bacteria. Such a situation has already been demonstrated for the arrangement of genes on the *Pseudomonas* chromosome (19); thus we should not be surprised if such novelty extends to the level of control of gene expression.

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